Injectable Cryogels for Biomedical Applications

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To prevent postoperative complications, there has been a substantial interest in designing syringe-injectable hydrogels. To date, cryogels remain the only viable option for preformed and large-scale hydrogels to be delivered through a conventional needle–syringe injection. Cryogels, a type of hydrogel with exceptional features, are fabricated at subzero temperatures. This process typically results in a biomaterial with a unique macroporous network, shape-memory properties, and exceptional flexibility allowing syringe injectability. These advanced biomaterials have been used for a number of biomedical applications, including tissue engineering, drug delivery, and more recently, immunotherapy. This review summarizes the recent progress on the design of injectable cryogels, their current limitations, and strategies to further improve their properties for translatability into the clinic.

How Cryogels Overcome the Limitations of Standard Injectable Hydrogels

For a number of biomedical applications, including cell therapy and tissue engineering, there is an increasing need to engineer advanced 3D scaffolds to provide structural and mechanical support for cells and facilitate tissue regeneration [1–3]. For the latter, these constructs need to mimic the complex physical and biochemical properties of the native extracellular matrix (ECM) (see Glossary) [4–6]. Additionally, the scaffolds must promote cell survival, proliferation, motility, and differentiation, as well as tissue integration within host tissues [7–9]. In this context, 3D scaffolds should be fabricated from biocompatible and resorbable polymers and have large interconnected macropores, ranging from 10 to 400 μm based on the targeted tissue. Furthermore, they should provide a physical framework as well as a large surface area to enhance cell housing and encourage tissue formation [10,11].

Hydrogels have been extensively used as polymeric scaffolds due to their high water content, biocompatibility, and physical properties similar to soft tissues (Figure 1, Key Figure) [12]. However, the small pore sizes (usually in the nanometer range) of conventional hydrogels have hindered their biomedical application due to limited cellular motion, cell spreading, and molecular diffusion of proteins, oxygen, and nutrients/waste products [13,14]. To address these challenges, porogens such as sacrificial particles and organic solvents have been investigated to increase hydrogel porosity [15,16]. However, due to insufficient pore interconnectivity and concerns over toxicity associated with incomplete porogen removal, their clinical application has been impeded. Alternatively, a simple and more eco-friendly technique known as cryogelation has recently attracted much interest as it produces macroporous hydrogels without the need to use toxic organic solvents [17]. Cryo-hydrogels (or cryogels) are typically formed in water (solvent) at subzero temperatures. When the solvent freezes, ice crystals form and subsequently expel the gel precursors (monomers, polymer, crosslinker, and initiator), which concentrate into an unfrozen phase (Figure 2). The cryopolymerization or gelation occurs around ice crystals generating a dense, highly crosslinked polymer network. When thawed, ice crystals leave behind a continuous and interconnected macroporous system (Box 1) [18,19]. These advanced hydrogels have recently

Highlights

Injectable cryogels were disclosed in 2012 as the first preformed large-scale hydrogels to be injected through a conventional hypodermic syringe, obviating the need for invasive surgical implantations.

Injectable cryogels have shape-memory properties and are reversibly compatible.

When syringe injected, shear-collapsed cryogels flow through conventional needles. Once released, cryogels instantly pop back to their initial shape and size.

Cryogels with their inherent interconnected macroporous network can host and/or deliver mammalian cells for tissue regeneration, cell transplantation, and in situ cell manipulation/reprogramming.

Biomolecules can be efficiently entrapped within the dense polymer walls of cryogels. Fine-tuning cryogel properties can control the spatiotemporal release of their payloads.

Enhancing cryogel properties will expand their potential in the biomedical field.

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drawn great attention for use in a wide variety of biomedical applications such as bioseparation, drug delivery, tissue engineering, and regenerative medicine [17].

Minimally invasive delivery of hydrogels is a critical aspect to bypass open surgery and associated postsurgical complications once these scaffolds are implanted in the body [18,20,21]. Therefore, engineering injectable hydrogels has become an active field of research (Figure 1) [22,23]. To enable injection, most in situ forming hydrogels are delivered in a liquid form that will subsequently solidify in the body [24,25]. Typically, the injected precursor gel solution forms a hydrogel via chemical (e.g., Michael-type addition reaction, disulfide bond formation, click chemistry, radical polymerization) or physical (e.g., ionic interactions, hydrogen bonding, hydrophobic interactions) crosslinking [26]. However, this strategy presents several limitations, including suitable gelation time, formation of gels with inadequate mechanical properties, toxicity, and the ability to protect the cargo of biomolecules or cells in complex biological environments [27]. Additionally, liquid precursor solutions may leak into surrounding tissues or dilute within the body fluids, which may not only limit hydrogel formation but also alter gel properties [21,22,28]. To overcome these limitations, it is essential to engineer solid (preformed) and well-characterized scaffolds capable of being injected, without any structural damage, through conventional syringes [17,18,27]. Shear-thinning hydrogels, a class of physically crosslinked gels that liquefy under shear stress and self-heal once injected, partly address these concerns [29,30]. Due to their impressive self-healing properties, shear-thinning hydrogels have shown minimal negative side effects once introduced in the body [31]. However, these gels are often associated with a nanoporous network, a nonmemorized geometry, and weak mechanical properties due to the fragile nature of physical crosslinking [29]. Therefore, engineering injectable preformed hydrogels with shape-memory features, improved mechanical properties such as flexibility, and with an open macroporous structure that retains its network intact upon injection would be an attractive alternative. Bencherif and colleagues disclosed in 2012 such injectable hydrogels, called cryogels, for the first time [18]. These particular cryogels, capable of recapitulating aspects of the native ECM, were easily injected manually through a conventional hypodermic needle. This technological advance has sparked since then massive interest in the field. These cryogels with unique attributes have created a new class of injectable materials applicable for a number of biomedical applications [17–19,32]. In this review, we discuss recent efforts in the development and applications of state-of-the-art injectable cryogel scaffolds in detail. Their inherent and unique features are a subject of considerable scientific research interest, which should accelerate and expand their potential for various hi-tech biomedical applications. To date, injectable cryogel scaffolds prepared from natural and synthetic polymers have been investigated in several biomedical applications, including cell therapy, drug delivery, biosensing, wound healing, and tissue engineering (i.e., bone, skin, neovascularization, neural, and adipose tissues). This review summarizes recent strategies, as well as challenges, to further improve their properties. Finally, a brief outlook on the future prospects of injectable cryogels is presented.

**Development and Applications of Injectable Cryogel Scaffolds**

Over the past decade, injectable cryogels have been extensively investigated for the minimally invasive implantation of 3D scaffolds. Both micro- and macroscale cryogels have been utilized to provide a porous construct in 3D, help protect encapsulated biological agents against degradation, as well as to control the delivery of mammalian cells and/or biomolecules to host tissues [33]. However, particle-based biomaterials may show inadequate retention at the injection site due to their small size [34]. This limitation increases the need for repeated injections, potentially leading to severe side effects and increased healthcare cost. To overcome these
limitations, macroscale cryogels are being increasingly investigated as they can create a confined and localized construct remaining at the injection site [33]. With their remarkable sponge-like macrostructural characteristics, combined with a set of unique features (e.g., shape-memory properties), injectable cryogels have emerged as a biomaterial of choice for a number of biomedical applications [17]. Their suitability for applications in tissue engineering and immunomodulation will be discussed in the next sections.

Hosting Cells within the Macroporous Framework of Cryogels
Injectable cryogels hold great promise for tissue engineering and immunomodulation [17,35]. Their macroporous structure and high porosity promote cell survival, adhesion, infiltration, and proliferation (Figure 3). The first published article on injectable cryogels clearly demonstrated their great potential as scaffolds for 3D cell culture and as a carrier for cell transplantation and drug delivery [18]. In this work, the cryogelation of methacrylated-alginate (MA-alginate) revealed new physical properties of cryogels, including shape-memory features and injectability. Additionally, this study showed the capacity of cryogels to control the sustained and long-term release of large biomolecules, such as bovine serum albumin and granulocyte-macrophage colony-stimulating factor (GM-CSF).

Depending on the application, injectable cryogels can be fabricated from natural and/or synthetic polymers. For example, Rezaee Yazdi and colleagues have demonstrated that cryogel scaffolds can be made out of natural polymers while retaining their intrinsic biological characteristics, even after cryotreatment [23]. Their hyaluronic acid (HA)-based cryogels displayed robust mechanical properties and injectability but lacked NIH-3T3 fibroblast cell adhesion; although gelatin-based cryogels exhibited weaker physical properties while promoting cell attachment, motility, and survival. However, when these two biopolymers were mixed together, HA-co-gelatin cryogels exhibited improved properties, combining the advantageous features of each polymer, including syringe injectability. In another study, Bruns and colleagues have engineered injectable cryogels made out of synthetic polymers to support cell growth and delivery [36]. They showed that their injectable polyethylene glycol (PEG)-based cryogels were cytocompatible. Additionally, they demonstrated that the physical properties could be effectively controlled, highlighting the ability of cryogels to be fine-tuned for a specific application.

Unlike hydrogels, cryogels cannot be loaded with cells prior to crosslinking as they will most likely not survive the process of cryopolymerization. Instead, cells are conveniently infused within the macroporous network of cryogels once fabricated, purified, and sterilized. Due to their ability to dissipate mechanical energy during compression, cell-laden cryogels are capable of protecting and retaining host cells upon injection [17]. These unique features make cryogels applicable for cell-related applications. For instance, Kim and colleagues demonstrated that heparin-co-gelatin cryogels could achieve high cell retention and survival following injection of NIH-3T3 fibroblasts. Due to these remarkable properties, injectable cryogels have also been used to restore blood flow by delivering fibroblasts in combination with endothelial growth factors [37]. Similarly, Bédouer and colleagues found that alginate and carboxymethyl-cellulose (CMC) cryogels infused with primary neurons not only maintained high cell adhesion but also protected the neuronal network during injection [15]. Furthermore, several approaches to functionalize cryogels with cell adhesion peptides (e.g., RGD, FGFRG) or ECM proteins (e.g., laminin, fibronectin, collagen) have been investigated to further enhance cell-matrix interactions and cell retention at the injection sites [18,36]. Unlike nanoporous in situ forming hydrogels, cells delivered with cryogels are associated with less physical restraints, facilitating cell motility and trafficking [38,39].
Although cell transplantation has many promising applications, tissue engineering often requires the delivery of more organized cell-based constructs. The technique of 3D printing has recently gained momentum as a promising approach to recreate complex tissues through layer-by-layer deposition [40]. Injectable cryogels can be used as a substrate onto which cell layers can be printed. Qi and colleagues engineered injectable composite cryogels to manufacture large 3D bioprinted adipose tissues [41]. These cryogels were fabricated from a combination of gelatin, HA, and PEG, and mimicked the soft mechanical properties of native adipose tissues. Next, human umbilical vein endothelial cells and human adipose-derived mesenchymal stromal cells (HADMSCs) were 3D printed onto these cryogels. These constructs promoted attachment, proliferation, and the spontaneous adipogenic differentiation of HADMSCs, resulting in capillary-like network formation. However, emulating the biomechanical features of soft tissues while enabling minimally invasive delivery and integration of the printed tissue constructs into host tissues remains a major challenge. Additive manufacturing of injectable cryogels can meet these specific requirements, but the technology of 3D cryoprinting is in its infancy and still remains quite challenging. Consistent subzero temperatures across the 3D-printed layers and controlled crosslinking kinetics are required to fabricate desirable injectable cryogels. Recently, some of these challenges were overcome when a microfluidic system was used to homogeneously mix the cryogel precursor solution prior to printing [42]. Bédueur and colleagues leveraged this technique to bioengineer cryogels suitable for neovascularization [27]. In this study, they investigated the effect of pore size on blood vessel formation from 3D printed CMC-based cryogels. When tested in mice, subcutaneous injection of these cryogels with the largest pore sizes (130 μm versus 16 μm) facilitated new blood vessel formation.

Other advantages of cryogels are their capacity to facilitate cellular infiltration and trafficking, as well as the controlled release of biomolecules. These attributes are of particular interest in the context of immunotherapy, where a construct can be used as a platform to manipulate immune cells in the body. Bencherif and colleagues have developed cryogel-based cancer vaccines to reprogram the immune system against melanoma [19]. Once subcutaneously injected into the body, these cryogel vaccines co-delivered irradiated tumor cells (antigens) and immunomodulatory factors (adjuvants and cytokines). Furthermore, these cryogel constructs enabled infiltration and activation of dendritic cells (DCs), the main regulator of the adaptive immune response [19]. Cryogel vaccines were also tested in the context of breast cancer [22]. In both studies, these cryogel vaccines delayed tumor onset and substantially improved survival (80%) in a prophylactic setting [19,22]. When tested therapeutically, cryogel vaccines strikingly induced regression of established melanoma [19].

In a different approach, Shah and colleagues developed injectable alginate-based cryogels to support the generation of T-cells in mice following hematopoietic stem cell (HSC) transplantation [43]. Once introduced in the body, these cryogels loaded with cell-instructive cues induced T lineage-specific differentiation of transplanted cells. Mice treated with these bone marrow-mimicking cryogels generated life-saving T cells from the transplanted HSCs faster than untreated animals [43].

**Injectable Cryogels Are a Minimally Invasive Platform for Biomedical Applications**

To prevent complications associated with surgical scaffold implantation, injectable cryogels are a viable alternative [18]. Zhao and colleagues fabricated injectable hemostatic cryogels for delivery into deep and bleeding wounds. Once in contact with blood, these cryogels expanded quickly and mitigated bleeding [44]. In comparison with commonly used gauze and gelatin hemostatic sponges, these cryogels exhibited improved hemostatic performance when tested in various extensively used in the biomedical and pharmaceutical fields.

**Hydrogel:** a 3D crosslinked network of polymer chains, capable of holding large amounts of water and body fluids due to their hydrophilic structure. Hydrogels are biomaterials of choice and have been extensively investigated and used in the biomedical field.

**Matrix metalloproteinases (MMPs):** a group of enzymes that are responsible for the degradation of most extracellular matrix proteins (e.g., collagen) during organogenesis, growth, and normal tissue turnover.

**Mesenchymal stromal cells (MSCs):** multipotent cells that can differentiate into a variety of cell types, including osteoblasts (bone cells), chondrocytes (cartilage cells), myocytes (muscle cells), and adipocytes (fat cells). MSCs are widely used for tissue regeneration and immunomodulation.

**Methacrylate (MA):** functional moiety containing a polymerizable vinyl group. MAs are often used to polymerize and crosslink monomers and/or polymers to form hydrogels.

**NIH-3T3:** a standard fibroblast cell line used in biomedical research.

**Platelet-rich plasma (PRP):** a blood-derived concentrate of platelet-rich plasma proteins and growth factors.

**Polyethylene glycol (PEG):** synthetic polymer with many applications, from industrial manufacturing to medicine.

**Polypyrrole:** heterocyclic conductive polymer.

**RGD:** Arg-Gly-Asp; sequence in fibronectin as the minimal integrin binding motif. RGD is used to promote both cell-substratum and cell-cell interactions.
mouse bleeding models. Furthermore, these cryogels also promoted wound healing in a rabbit model.

Injectable cryogels have also been considered for bone tissue regeneration, including the treatment of pathologic fractures. Bone defects often require invasive surgery and are difficult to treat [45]. To tackle these challenges, Lu and colleagues functionalized injectable alginate-based cryogels with platelet-rich plasma (PRP) [46]. Although incorporating PRP negatively impacted the physical features of cryogels, several cycles of freeze-thawing improved their porosity and compressibility. These PRP-loaded cryogels enhanced the proliferation of human bone osteosarcoma cells and induced their mineralization. Another strategy relied on the encapsulation of biphasic calcium phosphate (BCP) to bestow cryogels with osteoconductive and resorptive properties for bone remodeling. Abueva and colleagues demonstrated that chitosan-BCP composite cryogels (CSGs) were injectable and displayed good protein absorption, supporting preosteoblast cell attachment and proliferation [47]. Additionally, CSGs that were subcutaneously injected in rats exhibited good biocompatibility and promoted recruitment of polymorphonuclear leukocytes, cells involved in the first stage of fracture repair.

As the cryogel dimensions and shape can be preset prior to injection, the desired geometry can be easily achieved to match a particular defect. These properties are especially useful for applications in soft tissue reconstruction, in which hydrogels are typically injected beneath the skin. To demonstrate their potential as dermal fillers for soft tissue augmentation, Cheng and
colleagues subcutaneously injected heart-shaped HA-based cryogels in mice. These cryogels popped back to their original shape and size, and remained unaltered up to 30 days postinjection [48].

Minimally invasive delivery via syringe injection minimizes the risk of implantation-related complications (e.g., infections), which is a key consideration when designing biomaterials [44,49]. The biocompatibility of a material is defined from its interaction with the host tissue.
Capsule formation around implants indicates acute and/or chronic inflammatory responses [50]. For subcutaneously injected cryogels, host responses have been mixed: some cryogels induced capsule formation [27,51], while others did not [47,48]. These findings indicate that a number of important factors (e.g., type of polymer, polymer charge, sterilization method, degradation products) need to be taken into account when designing cryogels [49,52]. Unlike conventional hydrogels, injectable cryogels have recently been engineered to withstand autoclave sterilization [53]. This technique is expected to substantially improve their biocompatibility as reported by Villard and colleagues [53]. On the contrary, a subset of injectable cryogels have been designed purposely to stimulate immune cells in the context of cancer immunotherapy [6,19,22,43,51].

### Strategies to Enhance Properties of Injectable Cryogels

To further expand their applications in the biomedical arena, various strategies are being investigated to improve the properties of injectable cryogels. One of their main limitations is the insufficient control over the release of biomolecules, especially low molecular weight components. Due to their inherent high porosity, drug-loaded cryogels have often been associated with a burst release, limiting their potential as a drug delivery carrier [54,55]. To improve drug release kinetics, Koshy and colleagues hybridized cryogels with laponite nanoparticles (NPs) preloaded with immunomodulatory factors [54]. Unlike laponite-free cryogels, immobilizing laponite NPs in cryogels prevented the initial burst release. Additionally, varying laponite content further fine-tuned release kinetics from cryogels while preserving their syringe injectability. In another study, Bauleth-Ramos and colleagues encapsulated anticancer drug Nutlin-3a within acetalated dextran NPs in alginate cryogels. The encapsulation of Nutlin-3a within the NPs prevented their burst release from cryogels [56].
Biodegradation, an important element when designing scaffolds for tissue engineering, is necessary to facilitate new tissue formation and integration upon implantation. However, cryogel degradation is often challenging due to the synthetic (or semisynthetic) nature of polymers utilized in addition to a highly crosslinked and stable polymer network. For example, semisynthetic cryogels made out of HA remained unchanged for at least 30 days when subcutaneously injected in mice [48]. In a different study, heparin-co-gelatin cryogels were resistant to enzymatic degradation when subjected to collagenase treatment [37]. However, Koshy and colleagues developed gelatin-based cryogels that were susceptible to degradation when exposed to cell-secreted enzymes, including matrix metalloproteinases (MMPs) [51]. Interestingly, the release of GM-CSF from these cryogels boosted the recruitment of MMP-producing immune cells, resulting in their faster disintegration. This concept of cell-mediated degradation could be further investigated and leveraged to better control the degradation, as well as the release of biomolecules and drugs from cryogels. For example, a number of strategies could be explored to investigate whether concepts employed in the design of MMP-sensitive hydrogels could be implemented to injectable cryogels. These approaches are based on the use of MMP-cleavable crosslinkers or co-monomers to allow cell-mediated enzymatic degradation [52,57]. Alternatively, partially oxidized biopolymers (e.g., alginate, HA, dextran) and polyester derivatives [e.g., PEG-co-poly(glycolic acid)] could be utilized to tune the hydrolytic degradation rate of cryogels [58–62].

Electrically conductive scaffolds can help restore the electrical communication in biological systems. Recently, injectable cryogel bioelectronics have been developed by introducing
electrically conductive components. For instance, polypyrrole-conjugated elastin-co-gelatin cryogels hybridized with carbon nanotubes exhibited electrical conductance upon compression [63]. Additionally, incorporating iron-oxide NPs turned these hybrid cryogels magnetically responsive, making them remotely controllable. These conductive cryogels can be useful for neuromodulation and cardiac tissue engineering when electrical stimulations and mechanical softness are a prerequisite. In a different approach, conductive injectable cryogels have been used to promote wound healing. Zhao and colleagues developed quaternized chitosan-based cryogels hybridized with carbon nanotubes [44]. Once inoculated into wounds of mice and rabbits, these cryogels significantly enhanced wound healing and reduced blood loss. Furthermore, these chitosan-containing cryogels exhibited excellent antibacterial activity, which could further reduce the risks associated with infections and help patients heal faster.

**Improving Cryogel Injectability**

**Improving Injectability of Large-Scale or Bulk Cryogels**

The injectability of cryogels is a result of their reversibly collapsible and elastic structure. Their shape-memory properties are due to their crosslinking degree and mechanism, nature of polymers employed, and unique structural features such as interconnected macropores and dense polymer walls. To date, large-scale cryogels (with dimensions up to 8 × 8 × 1 mm) have been injected through 16-gauge (16G) needles. Although a 16G needle injection reduces invasiveness in comparison with surgical implantations, tissue damage can potentially be minimized to a greater extent when smaller needles are used. To reach that goal, cryogels require further optimization to exceed their compaction and improve injectability. One approach has been to reduce the polymer concentration. For instance, both the injectability and mechanics were tweaked by simply reducing the polymer content of gelatin-based cryogels, allowing their injection through 17G needles [37,51].

The importance of cryogel elasticity during injection was confirmed by Liu and colleagues. They showed that the incorporation of a rigid polypyrrole network within gelatin-co-HA cryogels altered their elasticity and ultimately injectability [63]. Furthermore, Qi and colleagues demonstrated that cryogel robustness and mechanical properties are improved when a 4-arm PEG crosslinker is used. As a result, the cryogels were reinforced and were able to be pushed through 16G needles [41].

While most injectable cryogels can be injected through large needles, injecting them through finer needles remains a major challenge. Shih and colleagues engineered tough alginate-based cryogels that simultaneously combine covalent and ionic crosslinking [22]. Unlike standard injectable cryogels that are typically covalently crosslinked, tough cryogels can be successfully injected through 18G needles. This type of cryogel exhibits high stretchability, in part due to reversible ionic crosslinking and self-healing properties. Another strategy to use thinner needles is simply based on injecting smaller cryogels. Bruns and colleagues investigated the syringe injectability of disc-shaped PEG-based cryogels while varying their overall diameters [36]. While all cryogels were successfully injected through 16G needles, they showed that smaller cryogels (diameters <2 mm) could also be injected through smaller 21G needles.

**Small-Scale Cryogels for Cell Therapy**

For some specific applications, using large needles (<18G) during injections may cause tissue injury, including bleeding and tissue disruption. The use of thinner needles (≥25G) is preferred for sensitive organs (e.g., brain) or tissues (e.g., nerves). Since bulk cryogels still cannot be injected through small-bore needles, micro-engineered cryogels (microcryogels) with diameters up to 600 μm have been investigated. Newland and colleagues fabricated ~300 μm diameter
microcryogels by crosslinking 4-arm PEG and heparin in a water-in-oil emulsion [64]. These microcryogels were able to remain intact when injected through a 27G needle and effectively maintained high cell viability when carrying neuron-like cells. Additionally, other approaches have been investigated to fabricate microcryogels. Liu and colleagues generated PEG-based microcryogels on microarray chips that could be optionally loaded with human MSCs [65]. In contrast to large-scale cryogels, microcryogels can facilitate a more homogeneous cell distribution following seeding, lower the risks of cell necrosis, and be injectable through 27G needles. Another advantage of microcryogels is their potential to fill in large and irregular tissue defects [66]. For example, injectable gelatin-based microcryogels have been used to specifically adhere to liver lesions via transglutaminase-mediated binding. Furthermore, when infused with MSCs, these constructs effectively treated mice with local liver injuries [67].

Despite several advantages, microcryogels do not create a well-defined macroscopic environment and may diffuse out of the injection sites postimplantation. These limitations make large-scale cryogels more appealing scaffolds. That being said, microcryogels offer a number of advantages over traditional microhydrogels due to their inherent porous structure. For instance, microcryogels made out of different polymers, such as PEG, gelatin, or alginate, are injectable through 27G needles, while their microhydrogel counterparts are not [65,68,69]. Additionally, Zeng and colleagues demonstrated that injecting MSCs with alginate along PEG-based microcryogels through 21G needles resulted in markedly increased cell viability when compared with alginate alone [70]. As microcryogels can effectively protect cells from shear stress-induced cell death experienced with narrow-bore needles, they have been applied for wound healing, intervertebral disc degeneration, bone repair, kidney disease, and ischemic limbs [69–73].

Concluding Remarks and Future Perspectives

Over the past decade, injectable cryogels have gained a rapid and wide interest in the biomedical arena. They have been utilized as drug/cell delivery carriers, 3D scaffolds for tissue engineering, dermal fillers in cosmetic surgery, and cell-instructive platforms for immunotherapy (Table 1). Their unique interconnected macroporous architecture makes them fit to host and protect mammalian cells during syringe-injection and provide a microenvironment favorable for cell delivery, cellular infiltration, and neovascularization. The minimally invasive delivery of scaffolds can obviate the risks associated with surgical implantations. To date, being the only preformed and injectable large-scale macroporous scaffolds with shape-memory properties, cryogels are set to advance the field, particularly in tissue engineering and immunotherapy.

Although significant advances in cryogel technology have been made, much more research is needed to address some of the current technical challenges. A major limitation of cryogels is their poorly controlled degradation behavior. Therefore, more strategies to fine-tune their resorption rates are needed. Additionally, the fast release of low molecular weight compounds (e.g., drugs, biomolecules) is another drawback across many cryogel scaffolds. One approach to slow down drug release kinetics could be the hybridization of cryogels with drug-loaded NPs. Furthermore, more effective surface grafting techniques (e.g., bioorthogonal conjugation) of biomolecules on cryogels should be investigated to enhance cell-matrix interactions. All of these approaches in combination with advanced additive manufacturing (e.g., 3D cryoprinting) are likely to facilitate the fabrication of cryogel-assisted tissues with organized microarchitectural features. These technological advances are expected to further enhance our ability to manipulate cells and to help us better understand how biomaterials

Outstanding Questions

Can large-scale cryogels potentially be injected through small-gauge (>21G) needles?

Can cryogels be fabricated with preset geometry and dimensions to match irregular defects? Would they fill in the space precisely?

Can release mechanisms and kinetics of therapeutic agents from cryogels be shaped to recruit targeted cells more effectively? Should cryogels be hybridized with functional materials to further regulate the release of their cargo? Can one achieve high level of control with on-demand drug release?

What are the optimal physical and biochemical properties of cryogels for tissue engineering? Can cryogels provide cell-instructive cues to enhance cell proliferation and differentiation for tissue regeneration?

What strategies can make cryogels more susceptible to degradation? Can the properties of cryogels be modulated to match tissue growth and new ECM formation?

Can 3D printed cryogels emulate the architectural complexity of native tissues and organs? Could they retain such complex 3D structures postinjection?

Can one controllably predict cryogel-host tissue interactions and prevent unwanted inflammation?

Can bioengineered cryogels instruct immune cells to facilitate tissue regeneration?

What is the required cryogel size to induce a protective immune response in humans? Can immunostimulatory cryogels be scaled up for widespread treatment?
### Table 1. Biomedical Applications of Polymeric Injectable Cryogels

<table>
<thead>
<tr>
<th>Application</th>
<th>Polymer</th>
<th>Crosslinking mechanism</th>
<th>Cryogel dimension needle size</th>
<th>Description</th>
<th>Refs</th>
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<tbody>
<tr>
<td>In vivo applications</td>
<td></td>
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<tr>
<td>Allogeneic hematopoietic stem cell transplantation</td>
<td>MA-alginate PEG</td>
<td>Covalent</td>
<td>8 × 8 × 1 mm 16G</td>
<td>Bone marrow-mimicking gels stimulated cell generation from stem cells&lt;br&gt;Cryogels restored T cell levels in immunodeficient mice after HSC therapy&lt;br&gt;Induced T cells increased mice survival</td>
<td>[43]</td>
</tr>
<tr>
<td>Cancer immunotherapy</td>
<td>MA-alginate</td>
<td>Covalent</td>
<td>4 × 4 × 1 mm 16G</td>
<td>Cryogel vaccines contain whole tumor cells, GM-CSF, and <strong>CpG oligodeoxynucleotide (CpG ODN)</strong>&lt;br&gt;Gels induce recruitment, antigen uptake, activation, and dispersion of DCs&lt;br&gt;Therapeutic vaccination increases mice survival in a melanoma model</td>
<td>[19]</td>
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<tr>
<td>Cancer immunotherapy, drug delivery</td>
<td></td>
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<tr>
<td>Cell/protein delivery</td>
<td>MA-gelatin</td>
<td>Covalent</td>
<td>Ø 5 mm, h 1 mm 16G</td>
<td>Spermine-modified acetalated dextran NPs, loaded with anticancer drug Nutlin-3a, were loaded into GM-CSF/CpG-containing cryogels&lt;br&gt;After peritumoral injection, drug release rate was fine-tuned, leading to immunogenic tumor cell death</td>
<td>[56]</td>
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<td>Cell/protein delivery</td>
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<tr>
<td>Tissue engineering, bone</td>
<td>Chitosan-gluconic</td>
<td>Physical</td>
<td>Not reported 23G</td>
<td>Calcium phosphate-containing cryogels promoted attachment of preosteoblasts&lt;br&gt;Optimized calcium phosphate concentration enhanced cellular infiltration in rats</td>
<td>[47]</td>
</tr>
<tr>
<td>Tissue engineering, liver</td>
<td>PEG-alginate-gelatin</td>
<td>Covalent</td>
<td>Ø 5 mm, h 1 mm 16G</td>
<td>Poly(N-isopropylacrylamide) and HepG2 cells were incorporated in cryogels to form a human liver microtissue&lt;br&gt;A human ectopic liver was successfully established after peritoneal injection into mice</td>
<td>[84]</td>
</tr>
<tr>
<td>Tissue engineering, soft tissue</td>
<td>MA-HA</td>
<td>Covalent</td>
<td>Ø 5 mm, h 2 mm 16G</td>
<td>Cryogels maintained skin firmness and preset geometry up to 30 days following injection in mice&lt;br&gt;Cryogels did not induce an inflammatory response in mice&lt;br&gt;Cryogels promoted neovascularization</td>
<td>[48]</td>
</tr>
<tr>
<td>Tissue engineering, vascularization</td>
<td>CMC</td>
<td>Covalent</td>
<td>30 × 40 mm 0.8 mm catheter</td>
<td>3D cryoprinting can control cryogel porosity and cell density&lt;br&gt;Cryoprinted gels protected host cells during injection&lt;br&gt;Tissue infiltration is dependent on scaffold porosity&lt;br&gt;Larger pores enhanced vascularization in mice</td>
<td>[27]</td>
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<tr>
<td>Tissue engineering, vascularization</td>
<td>Gelatin Heparin</td>
<td>Covalent</td>
<td>Ø 5 mm, h 3 mm 17G</td>
<td>Heparin allows sustain release of VEGF from cryogels&lt;br&gt;Low polymer content improved injectability and retention of fibroblasts at the injection site&lt;br&gt;Cell/VEGF delivery restored blood flow in a hind limb ischemia model</td>
<td>[37]</td>
</tr>
<tr>
<td>Tissue engineering, wound healing</td>
<td>MA-glycidyl Quaternized chitosan</td>
<td>Covalent</td>
<td>Ø 5 mm, h 20 mm</td>
<td>Cryogels hybridized with carbon nanotubes increased electrical conductivity</td>
<td>[44]</td>
</tr>
</tbody>
</table>
Lastly, concerns over the cost, manufacturability, reproducibility, and scalability of cryogels still remain. Standardizing cryogel manufacturing is expected to address some of these obstacles. Additionally, the size of injectable cryogels could be a barrier when moving from preclinical research into clinical practice, where larger scaffolds are typically required for humans. To that end, several strategies are currently being explored, such as looking at various crosslinking mechanisms and cryogel geometries, as well as optimizing the macrostructural features of cryogels (e.g., pore size, pore connectivity, pore orientation, toughness, flexibility). Looking into the future, engineering more compressible or self-healing cryogels, designing smart cryogels sensitive to external stimuli, and fabricating cryogels with more defined macrostructural features should broaden their biomedical utility and expedite their clinical translatability.

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